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The Hyperpolarization-Activated HCN1 Channel Is Important for Motor Learning and Neuronal Integration by Cerebellar Purkinje Cells

Matthew F. Nolan,[1] Gaël Malleret,[1] Ka Hung Lee,[5] Emma Gibbs,[1] Joshua T. Dudman,[1] Bina Santoro,[1] Deqi Yin,[1] Richard F. Thompson,[5] Steven A. Siegelbaum,[1,2,4] Eric R. Kandel,[1,3,4] and Alexei Morozov[1,6]

[1]Center for Neurobiology and Behavior
[2]Department of Pharmacology
[3]Departments of Physiology, Biochemistry and Biophysics, and Psychiatry
[4]Howard Hughes Medical Institute
Columbia University
New York, New York 10032
[5]Program in Neuroscience
The University of Southern California
3641 Watt Way
Los Angeles, California 90089
[6]Unit of Behavioral Genetics
National Institute of Mental Health
Bethesda, Maryland 20892

Summary

In contrast to our increasingly detailed understanding of how synaptic plasticity provides a cellular substrate for learning and memory, it is less clear how a neuron's voltage-gated ion channels interact with plastic changes in synaptic strength to influence behavior. We find, using generalized and regional knockout mice, that deletion of the HCN1 channel causes profound motor learning and memory deficits in swimming and rotarod tasks. In cerebellar Purkinje cells, which are a key component of the cerebellar circuit for learning of correctly timed movements, HCN1 mediates an inward current that stabilizes the integrative properties of Purkinje cells and ensures that their input-output function is independent of the previous history of their activity. We suggest that this nonsynaptic integrative function of HCN1 is required for accurate decoding of input patterns and thereby enables synaptic plasticity to appropriately influence the performance of motor activity.

Introduction

Studies of learning and memory have focused on an anatomical analysis of the neuronal circuitry underlying learned behaviors and on the importance within these circuits of activity-dependent, long-term modification of synaptic function (Medina et al., 2002; Milner et al., 1998). By contrast, relatively little is known about the significance for learning and memory of nonsynaptic properties, such as the intrinsic excitability of neurons within the neural circuits that mediate modifications of behavior (Hansel et al., 2001). We focus on one particular molecular component of neuronal electrical activity, the HCN1 channel, and its role in motor learning. Using both generalized and regional knockouts of HCN1, we explore its contribution to the electrophysiological properties of cerebellar Purkinje neurons and to forms of motor learning in which these neurons participate.

Plasticity within the cerebellum contributes to learning of complex temporal and spatial coordination of muscle activity that underlies the performance of motor skills (Doya, 2000; Hikosaka et al., 2002; Thach, 1998). Contextual and error signals important for motor behavior converge onto Purkinje cells via parallel and climbing fiber inputs. The Purkinje cells are inhibitory neurons that provide the sole output of the cerebellar cortex. For synaptic plasticity within the cerebellar cortex to contribute to learning of motor skills, it must cause a change in cortical output via Purkinje cells. Classical theories of cerebellar function emphasize the importance for learning of plastic changes in parallel fiber synapses when they are activated coincidentally with climbing fiber inputs (Albus, 1970; Marr, 1969). Indeed, such coincident activity induces long term depression of parallel fiber synapses, and considerable progress has been made toward elucidating the molecular basis of this form of plasticity (Ito, 2002). However, it is unclear how the nonsynaptic electrophysiological properties of the cerebellar circuit influence learning of motor skills.

A key nonsynaptic property of cerebellar Purkinje cells is the ability to fire spontaneous action potentials at frequencies of approximately 10–100 Hz (Häusser and Clark, 1997; Nam and Hockberger, 1997; Raman and Bean, 1999; Thach, 1968). Changes in spike activity induced by motor learning have been characterized in one well-studied form of motor learning, eye blink conditioning, in which an animal learns to associate a conditioned stimulus (e.g., a tone) with a noxious unconditioned stimulus (e.g., an air puff to the eye) that elicits an eye blink (Kim and Thompson, 1997; Medina et al., 2002). After learning, the conditioned stimulus initiates a pause in Purkinje cell spiking, which permits an increase in firing of target neurons in the deep cerebellar nuclei (Hesslow and Ivarsson, 1994). This in turn initiates the eye blink response (McCormick et al., 1982; Medina et al., 2002). Modification of Purkinje cell spike output is also associated with changes in the gain of the vestibulo-occular reflex (Raymond and Lisberger, 1998) and with changes in the coordination of limb movements (Gilbert and Thach, 1977). How the precise patterns of electrical activity generated by specific ion channels contribute to learning during these or other behaviors is not understood.

The hyperpolarization-activated, cyclic nucleotide-regulated nonselective cation (HCN) channels (Robinson and Siegelbaum, 2003) provide an interesting target for exploring the relationship between the electrical activity of neurons and behavior. These channels are active at membrane potentials important for integration of neuronal activity and are encoded by four genes (HCN1–4) with specific patterns of expression throughout the brain and in the heart (Kaupp and Seifert, 2001; Santoro et al., 2000). HCN1 is expressed at high levels in both the

*Correspondence: erk5@columbia.edu

Purkinje and the basket cells of the cerebellar cortex, suggesting it might have an important role in cerebellar function. Indeed, hyperpolarization-activated currents (I_h) with kinetic properties similar to those of recombinant HCN1 channels have been recorded from cerebellar Purkinje neurons (Crepel and Penit-Soria, 1986; Li et al., 1993; Roth and Häusser, 2001; Williams et al., 2002) and basket cells (Saitow and Konishi, 2000; Southan et al., 2000). However, it is unclear how suggested cellular functions of I_h, such as pacemaking of spontaneous action potential firing (Crepel and Penit-Soria, 1986; Li et al., 1993), shaping the waveform of dendritic synaptic potentials (Roth and Häusser, 2001), or preventing membrane potential bistability (Williams et al., 2002), may contribute to aspects of motor learning.

To address these issues, we have generated two lines of mice in which functional HCN1 channel subunits were deleted through homologous recombination. In one mouse line, the HCN1 channel was knocked out in all cells. In the other mouse line, the knockout was restricted to the forebrain. These two lines of HCN1 knockout mice have allowed us to study the function of this channel in forms of motor learning on the one hand, and in Purkinje cells that are the sole output of cerebellar cortical pathways that contribute to motor learning on the other. We find that HCN1 is important for learning and memory of motor behaviors involving relatively rapid, repeated, and coordinated movements and is required for history-independent integration of inputs by cerebellar Purkinje cells. We suggest a model whereby HCN1 stabilizes the input-output properties of Purkinje cell spiking during repetitive motor behaviors, thereby enabling plasticity in the cerebellar cortex to modulate the performance of repetitive motor activity.

Results

Generation of Mice with Complete and Restricted Knockout of HCN1

The HCN1 gene was inactivated by deleting the exon encoding the P region and S6 transmembrane domain. Conditional and conventional knockouts were generated using the three loxP site strategy (see Experimental Procedures and Supplemental Figure S1 at http://www.cell.com/cgi/content/full/115/5/551/DC1). Homozygous mutant, complete knockout ($HCN1^{-/-}$), and floxed ($HCN1^{f/f}$) mice appeared with the expected Mendelian frequency and did not differ from their wild-type littermates in overall health and longevity. Forebrain restriction of the HCN1 deletion was obtained by crossing transgenic mice expressing Cre-recombinase under the control of the CaMKIIα-promoter (line R1ag#5 [Dragatsis and Zeitlin, 2000]) with the floxed animals (see Experimental Procedures) to generate $HCN1^{f/f,cre}$ mice.

The molecular and biochemical analysis of the HCN1 knockout mice is described in detail in Supplemental Figures S1 and S2. Neither HCN1 protein nor mRNA encoding the pore and S6 transmembrane domain were detected from brains of $HCN1^{-/-}$ mice. HCN1 protein and mRNA encoding the P region and S6 transmembrane domain were detected in the cerebellum of $HCN1^{f/f,cre}$ mice, but were absent from the hippocampus and neocortex. We did not find any change in the expression level of HCN2 or HCN4 proteins in $HCN1^{-/-}$ animals; however, there was a consistent small increase in the expression of HCN3 protein. The significance of the latter finding is unclear, especially since HCN3 does not express currents in heterologous systems.

We found no difference in the anatomical properties of brains from $HCN1^{+/+}$ and $HCN1^{-/-}$ mice, including hindbrain regions involved in motor control (Supplemental Figure S3), nor did we find any effect of HCN1 knockout on the properties of synaptic inputs to cerebellar Purkinje cells. Thus, miniature inhibitory currents (Supplemental Figure S4 and Supplemental Table S1), climbing fiber responses (Supplemental Figure S5), and parallel fiber responses (Supplemental Figure S6) were similar in both groups of mice, indicating that HCN1 is not required for fast synaptic input to Purkinje neurons.

Mice with Knockout of HCN1 Have Normal Basic Motor Functions

We investigated the role of HCN1 in forms of motor learning that involve the cerebellum. It was therefore important to first determine if knockout of HCN1 causes a deficit in general motor ability. All $HCN1^{+/+}$ ($n = 4$) and $HCN1^{-/-}$ ($n = 3$) mice examined had normal eye blink, ear twitch, rolling, and righting reflexes. In a hot water tail flick test, $HCN1^{-/-}$ mice had a withdrawal latency (2.25 ± 0.48 s, $n = 4$) similar to $HCN1^{+/+}$ mice (2.29 ± 0.36, $n = 7$, $p = 0.95$). In addition, when $HCN1^{+/+}$ ($n = 4$) and $HCN^{-/-}$ mice ($n = 7$) were tested on a rotarod at a constant speed of 5 rpm, both groups remained on the rotarod for the maximum time of 300 s (data not shown). Thus, knockout of HCN1 does not appear to impair basic motor functions.

HCN1 Knockout Causes a Learning Deficit in a Visible Platform Water Maze Task

To investigate more complex behavior, mice were trained in a water maze to find a visible submerged platform marked by a flagpole (visible platform), prior to testing spatial memory with a hidden submerged platform (Figure 1) (Morris et al., 1982). The visible platform task enables testing for behavioral impairments unrelated to spatial memory. The performance of $HCN1^{+/+}$ and $HCN1^{-/-}$ mice on the first trial was similar, indicating that HCN1 is not important for naïve behavior in this task. However, with subsequent training, there was a greater reduction in the latency to reach the platform for $HCN1^{+/+}$ compared with $HCN1^{-/-}$ mice, indicating an impairment in the ability of the $HCN1^{-/-}$ mice to learn how to swim to the visible platform (Figure 1A). Analysis of the swim trajectories indicated that whereas $HCN1^{+/+}$ mice learned to swim directly to the platform, $HCN1^{-/-}$ mice maintained a tendency to swim in loops and spent more time closer to the sides of the pool (increased thigmotaxis), similar to the behavior of both groups on the first trial (Figures 1C and 1D). Swimming speed (Figure 1B) and floating time (not shown) were similar in both groups of mice, indicating the deficit was not due to a general motor or emotional impairment. Spatial memory was tested after training the mice to locate a submerged hidden platform. During a probe trial, the preference of $HCN1^{-/-}$ mice for the training quadrant was similar to $HCN1^{+/+}$ mice, indicating that they do

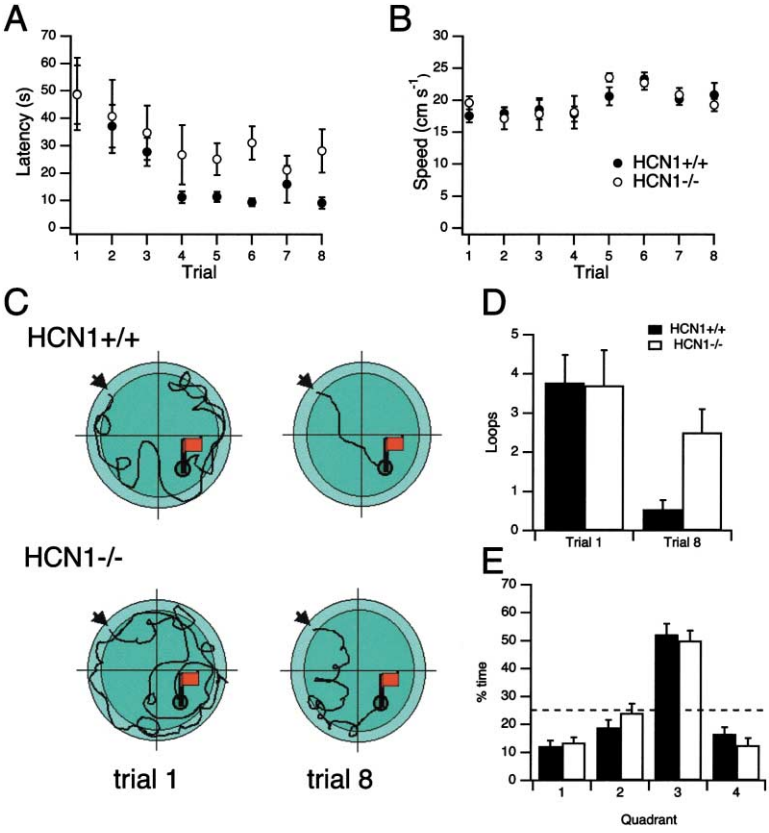

Figure 1. HCN1$^{-/-}$ Mice Are Impaired in Learning to Swim to a Visible Platform

(A) The latency to reach the visible platform as a function of trial number.

(B) Swimming speed as a function of trial number.

(C) Examples of paths followed to the visible platform by HCN1$^{+/+}$ (top) and HCN1$^{-/-}$ (bottom) mice on trials 1 (left) and 8 (right).

(D) The number of loops swum is similar on trial 1, but shows a greater reduction with training for HCN1$^{+/+}$ compared to HCN1$^{-/-}$ mice.

(E) Percent of time spent in a given quadrant during a probe trial following training in a hidden platform task during which the platform was present in quadrant 3. Dashed line shows percent time expected in each quadrant if no learning occurs.

not have any deficit in spatial localization and memory (Figure 1E). Altogether, these results suggest that knockout of HCN1 leads to impairment in some form of learned motor coordination required for swimming directly to a target in the pool.

Knockout of HCN1 Impairs Rotarod Learning

To further investigate motor performance and learning, we used a rotarod test. Mice were trained over three days to balance on an accelerating rod and on the fourth day were tested at various rotation speeds. HCN1$^{+/+}$ and HCN1$^{-/-}$ mice performed similarly on the first training session ($p = 0.406$). With subsequent training, the latency at which HCN1$^{+/+}$ mice fell from the accelerating rod increased almost 3-fold, from 71.4 $\pm$ 11.5 s to 206.1 $\pm$ 21 s, indicating learning of the motor skills required to balance on the rod ($p < 0.0001$). By contrast, the HCN1$^{-/-}$ mice showed only a relatively modest increase in their latency to fall, from 60.1 $\pm$ 17.3 s to 92.5 $\pm$ 18.6 s ($p = 0.02$) (Figure 2A). Following the training period, test performance was significantly worse in HCN1$^{-/-}$ compared to HCN1$^{+/+}$ mice at speeds above 20 rpm ($p < 0.05$) (Figure 2B). Expression of HCN1 therefore appears to be required not for basal motor coordination, but for learning of the motor skills which enable mice to balance on the rotating rod.

The rotarod learning deficit in HCN1$^{-/-}$ mice may be due to the absence of HCN1 from forebrain structures. We therefore compared rotarod performance of HCN1$^{f/f,cre}$ mice, which have knockout of HCN1 restricted to the forebrain, with that of HCN1$^{f/f}$ mice, which show normal expression of HCN1. During training, there was a similar improvement of performance for both groups of mice. The mean latency for HCN1$^{f/f}$ mice to fall from the accelerating rod increased from 146.3 $\pm$ 21.7 to 271.2 $\pm$ 14.3 s ($p < 0.0001$); for HCN$^{f/f,cre}$ mice, there was an increase in the latency from 99.5 $\pm$ 19.4 to 243.9 $\pm$ 18.3 s ($p < 0.0001$) (Figure 2C). The apparent trend in the HCN1$^{f/f,cre}$ mice toward a lower latency compared to HCN1$^{f/f}$ mice was not statistically significant ($p = 0.251$, ANOVA effect of genotype). During testing, there was no significant difference between the two groups at any rotation speed ($p > 0.3$) (Figure 2D). These data demonstrate that HCN1 expression in forebrain regions is not required for learning the rotarod task.

The Rotarod Learning Impairment in HCN1 Knockout Mice Is Greater at Faster Speeds

The rotarod deficit could indicate a general impairment in motor learning or may be more specific to higher rotarod speeds. To distinguish between these possibilities, we examined a second group of mice trained to balance on a rotarod turning at constant speeds (Figure 2E). At 14 rpm, there was initially no difference between the performance of HCN1$^{+/+}$ and HCN1$^{-/-}$ mice (trial 1, $p = 0.24$). However, the HCN1$^{+/+}$ mice were able to learn to balance on the rotarod ($p < 0.001$), whereas HCN1$^{-/-}$ failed to show a significant effect of training ($p = 0.65$). At a slower speed of 12 rpm, HCN1$^{-/-}$ mice did show an improvement in performance over 8 trials ($p < 0.05$), indicating that HCN1$^{-/-}$ mice are able to learn to balance on the rotarod at slower speeds. Importantly, when the

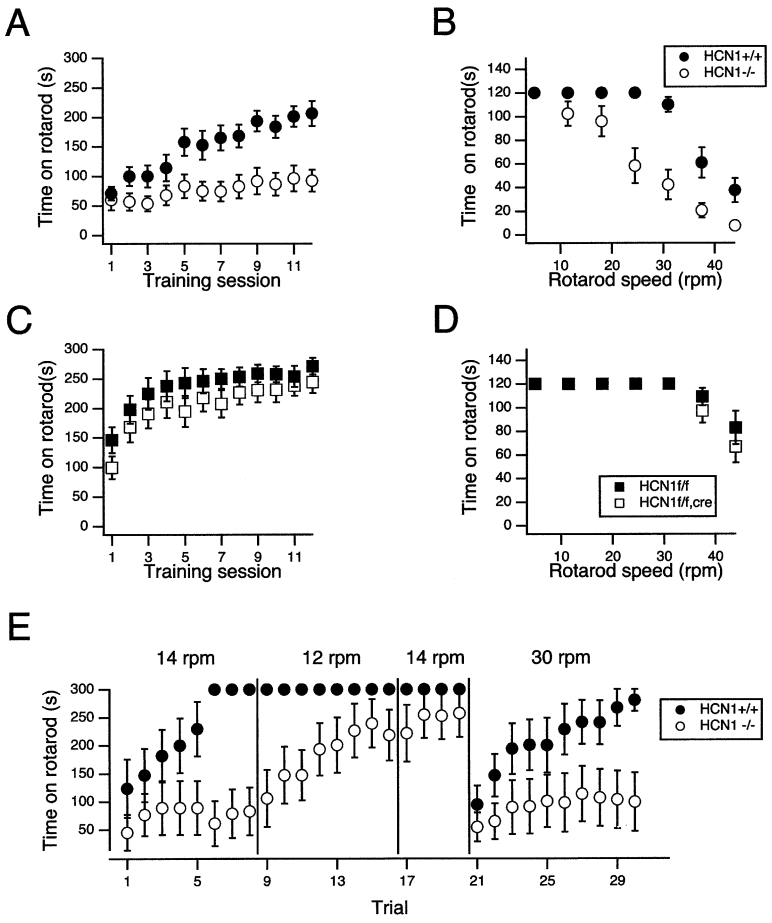

Figure 2. Rotarod Learning Is Impaired in HCN1$^{-/-}$ Mice

(A) Time that mice remained on an accelerating rotarod before falling as a function of training session. ANOVA indicated a significant effect of genotype ($p < 0.005$) and trial ($p < 0.0001$). Closed circles represent HCN1$^{+/+}$ ($n = 13$) and open circles HCN1$^{-/-}$ mice ($n = 11$).

(B) Time mice remained on the rotarod when tested at constant speeds between 5 and 44 revolutions per minute. There was a significant difference between genotypes ($p < 0.05$) at rotarod speeds of 22–44 rpm.

(C and D) Data as for (A) and (B), except that experiments are with HCN1$^{f/f,cre}$ mice, in which HCN1 knockout is restricted to the forebrain (open squares, $n = 13$), and with HCN1$^{f/f}$ litter mates as controls (closed squares, $n = 12$). ANOVA indicated a significant effect of trial ($p < 0.0001$), but no significant effect of genotype ($p = 0.403$) during training. During testing there was no significant difference ($p > 0.3$) between genotypes at any speed.

(E) Time that HCN1$^{+/+}$ ($n = 8$) and HCN1$^{-/-}$ ($n = 7$) mice remained on a rotarod turning at a constant speed as a function of trial number. The rotarod speed is 14 rpm during trials 1–8, 12 rpm during trials 9–16, 14 rpm during trials 17–30, and 30 rpm during trials 21–32. Labels as for (A).

HCN1$^{-/-}$ mice were retested for four trials at 14 rpm, they were now able to balance on the rod at this speed, suggesting the initial deficit involved impaired learning of the task, and this could be partially overcome by training at lower speeds. Subsequent testing at a more demanding speed of 30 rpm did not reveal an initial difference in the performance of the two groups of mice, supporting the conclusion that basal motor coordination is not effected by knockout of HCN1. However, during the 12 trials at 30 rpm, HCN1$^{+/+}$ mice greatly increased the time they could balance on the rod ($p < 0.001$), whereas HCN1$^{-/-}$ showed only a small improvement ($p = 0.45$). These data strongly suggest that the rotarod deficit is due to a specific impairment in learning and memory of relatively fast, coordinated movements.

HCN1 Knockout Mice Show Altered Timing of the Conditioned Eyelid Response

The importance of the cerebellum for learning motor skills and the high expression of HCN1 in the cerebellar cortex suggest that the motor learning deficits in HCN1$^{-/-}$ mice may be explained by changes in cerebellar function. Classical eyelid conditioning is a form of associative motor learning that has been studied extensively as a model for cerebellar dependent learning (Hansel et al., 2001; Kim and Thompson, 1997; Medina et al., 2002). We examined the consequences of HCN1 knockout for eye blink conditioning to determine if the synaptic mechanisms underlying cerebellar-dependent learning and memory are intact. There was no significant difference in acquisition or extinction of the conditioned response between HCN1$^{+/+}$ and HCN1$^{-/-}$ mice (Figures 3A and 3B), indicating that HCN1 is not required for synaptic plasticity underlying cerebellar learning or extinction. However, examination of the latency to the peak of the conditioned response revealed that knockout of HCN1 modified the timing of the conditioned response ($p < 0.003$) (Figures 3C and 3D), due to an increase in the number of responses with short latencies. Cerebellar cortical lesions can also result in shorter latency conditioned responses (McCormick and Thompson, 1984), suggesting impaired cerebellar cortical function in the HCN1$^{-/-}$ mice.

The deficits in learning during the visible platform and the rotarod tasks, together with the modification of response latencies during eyelid conditioning (cf. Chen et al., 1995; Shibuki et al., 1996), indicate that HCN1 may be important for motor learning and memory underlying relatively fast, repeated execution of coordinated movements, but not for association of unconditioned and conditioned stimuli. To determine what cellular mechanisms might account for these deficits, we focused our investigation on the contribution of HCN1 to the integrative properties of Purkinje cells. These neurons, which express high levels of HCN1 (Santoro et al., 2000), are the sole output neurons of the cerebellar cortex and

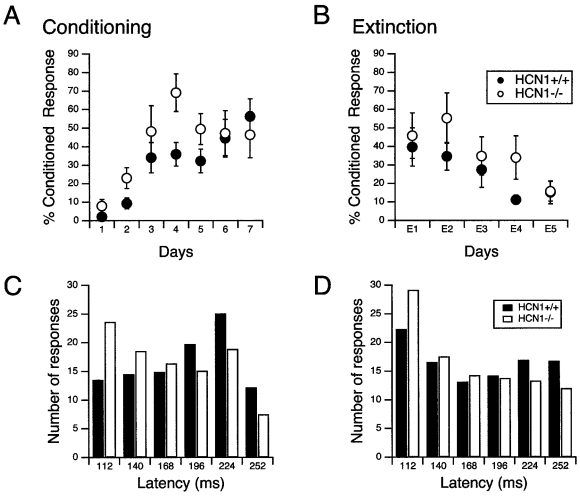

Figure 3. HCN1 Is Not Required for Eyelid Conditioning

(A and B) The percentage of trials, during conditioning (A) and extinction (B), on which a conditioned response to the tone occurred, as a function of trial number, was similar for HCN1$^{+/+}$ (n = 12) and HCN1$^{-/-}$ mice (n = 12).

(C and D) Comparison of the latency from the onset of the tone to the peak of the conditioned response during conditioning (C) and extinction (D) revealed an increase with knockout of HCN1 in the number of responses occurring at short latencies.

therefore any effect of HCN1 knockout on their spike output is likely to alter the contribution of the olivocerebellar system to behaviors involving motor learning.

HCN1-Mediated I_h in Cerebellar Purkinje Cells

Pharmacologically isolated hyperpolarization-activated currents were recorded at room temperature (see Experimental Procedures). In Purkinje cells from HCN1$^{+/+}$ mice, hyperpolarization to voltages negative to −60 mV activated a prominent I_h (Figure 4A). By contrast, in Purkinje cells from HCN1$^{-/-}$ mice, I_h was greatly reduced (Figure 4B). Comparison of tail currents revealed an approximately 10-fold decrease in I_h (Figure 4C), confirming the loss of functional channels and indicating that HCN1 is the major determinant of I_h in this cell type. A large I_h was recorded from HCN1$^{f/f,cre}$ and HCN1$^{f/f}$ mice, indicating that HCN1 was not deleted from cerebellar Purkinje cells in the forebrain-restricted knockout mice (Figure 4D–4F).

To obtain an accurate picture of the steady-state membrane properties of Purkinje cells, we examined their current-voltage relationship under voltage clamp conditions (Figure 5). This and subsequent experiments were conducted at 32–34°C in standard recording solutions (see Experimental Procedures). In contrast to HCN1$^{+/+}$ mice (Figure 5A), the membrane current in cells from HCN1$^{-/-}$ mice showed little dependence on voltage at potentials from −50 mV to −70 mV, reflecting a reduced slope conductance (i.e., the change in membrane current divided by the change in membrane potential) that was close to or less than zero (Figure 5B). Thus, whereas in HCN1$^{+/+}$ mice small deviations in the membrane potential are opposed by changes in membrane current, and therefore the membrane potential will be relatively stable, similar deviations in the membrane potential of HCN1$^{-/-}$ mice are not opposed (and in regions of negative conductance will be facilitated) by changes in membrane current, and the membrane potential will therefore become unstable (see Koch, 1999 for discussion). Comparison of average membrane currents (Figure 5C) and slope conductance (Figure 5D) confirmed that HCN1 prevents the emergence of this region of low or negative membrane conductance in Purkinje cells.

What is the mechanism underlying the low slope conductance in cerebellar Purkinje cells? In both HCN1$^{+/+}$ and HCN1$^{-/-}$ mice, the slope conductance was increased when sodium channels were blocked with tetrodoxin (TTX) (Figure 5E and 5F), indicating that activation of the TTX-sensitive resurgent sodium current (Raman and Bean, 1997, 1999) causes the low or negative membrane conductance. These data suggest that generation of spontaneous action potentials or integration of current inputs, at membrane potentials at which the membrane conductance was lowest (−50 to −70 mV), may become unstable in the absence of HCN1. We therefore investigated the consequences of HCN1 deletion for the integrative properties and spike output of Purkinje cells.

HCN1 Is Not Required for Spontaneous Spiking in Cerebellar Purkinje Cells

Although in some cells I_h acts as a pacemaker of spontaneous activity (Pape, 1996), we find that the mean spontaneous spike frequency is similar for Purkinje cells from HCN1$^{+/+}$ (46.8 ± 4.8 Hz) and HCN1$^{-/-}$ mice (48.5 ± 4 Hz, $p > 0.75$) (Figure 6A). Action potential threshold, rise-time, half-width, peak depolarization, and peak after hyperpolarization also were similar in both groups of mice (see Figure 6). The reduced membrane conductance in the absence of HCN1 could lead to spontaneous bistability, a switching between two stable states, of the Purkinje cell membrane potential, as is observed following pharmacological blockade of I_h (see Williams et al. 2002). However, Purkinje cells from HCN1$^{-/-}$ mice did not demonstrate spontaneous bistable behavior, although in some cells, when the membrane was hyperpolarized to ∼ −75 mV by constant current injection, bistable-like transitions from silent to spiking states could occur either spontaneously or, more often, in response to brief current steps (data not shown).

HCN1 Contributes to Integrative Properties of Cerebellar Purkinje Cells at Subthreshold Potentials

Although HCN1 is not required for spontaneous spiking, it may be involved in integration of hyperpolarizing inputs. We therefore examined responses of Purkinje cells to a series of negative current steps. In both groups of mice, spike frequency was reduced with increasing current step amplitude (Figure 6B and 6C), with no significant difference in the relationship between steady-state spike frequency and current (Figure 6D). In addition, the minimal current required to abolish spontaneous spiking was not altered significantly by knockout of HCN1 (HCN1$^{+/+}$ −235 ± 29.7 pA, HCN1$^{-/-}$ −185.4 ± 18.9 pA, $p = 0.16$). Therefore, HCN1 does not appear to be an

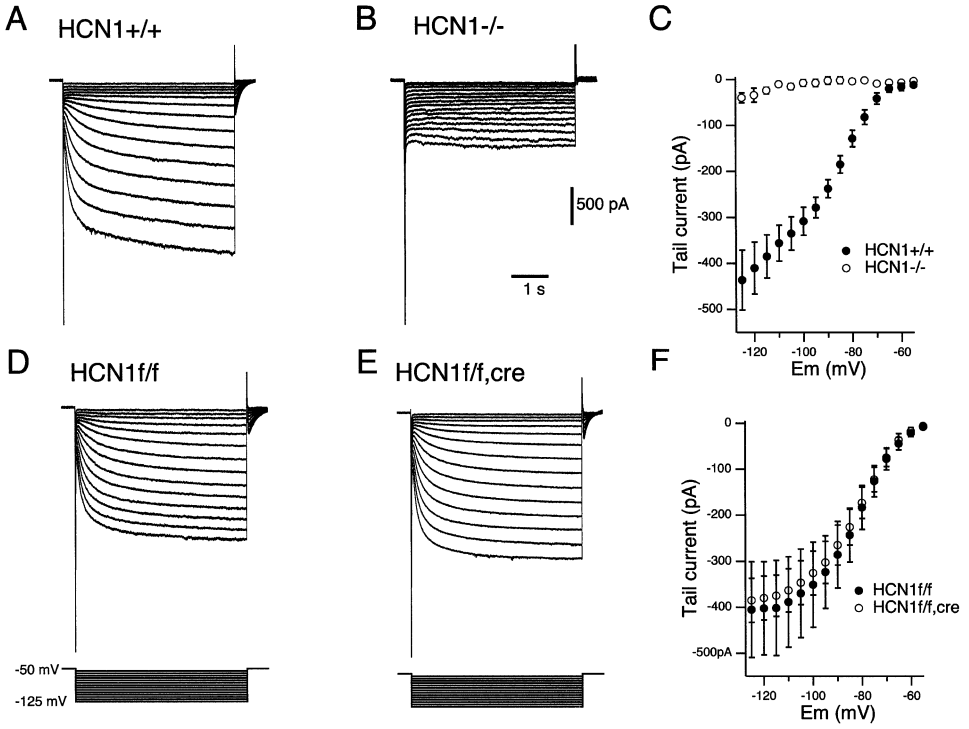

Figure 4. I_h in Cerebellar Purkinje Cells

(A–C) I_h in Purkinje cells from a HCN1$^{+/+}$ mouse (A) and a HCN1$^{-/-}$ mouse (B). Currents are in response to 5 s voltage steps to between −55 mV and −125 mV in 5 mV increments from a holding potential of −50 mV. (C) Mean tail currents recorded upon return to −50 mV, plotted against the preceding test potential.

(D–F) I_h in Purkinje cells from HCN1$^{f/f}$ (D) and HCN1$^{f/f,cre}$ mice (E) was similar, and there was no difference in tail currents (F). The voltage protocol is shown below traces in (D) and (E).

important determinant of the steady-state input-output properties of cerebellar Purkinje cells.

HCN1 does, however, influence the relationship between hyperpolarizing current and membrane potential at voltages negative to the threshold for spontaneous spiking. The modal membrane potential (see Experimental Procedures), during current steps whose amplitude was insufficient to abolish spontaneous spiking, was relatively independent of injected current and was similar in both HCN1$^{+/+}$ and HCN1$^{-/-}$ mice. During current steps large enough to abolish spontaneous spiking, the level of membrane hyperpolarization increased with the amplitude of the injected current and was markedly altered by knockout of HCN1 (Figures 6B and 6C). The smallest negative current step sufficient to abolish spiking hyperpolarized the membrane of HCN1$^{+/+}$ Purkinje cells to an average membrane potential of -61.7 ± 1.8 mV. In contrast, during the corresponding responses from HCN1$^{-/-}$ mice, the membrane hyperpolarized to -73.3 ± 1.5 ($p < 0.001$). In all Purkinje cells from HCN1$^{+/+}$ mice, the membrane potential of the smallest step that abolished spiking was less than 6 mV negative to that of the largest step during which spiking occurred, whereas in all cells from HCN1$^{-/-}$ mice, this difference was greater than 10 mV. The average steady-state I-V relationship was also steeper in the absence of HCN1 (Figure 6E), as expected from the voltage clamp I-V relationship (see Figure 5). Thus, although HCN1 does not appear to contribute to the steady-state firing properties of Purkinje cells, it is an important determinant of the steady-state relationship between current and membrane potential at voltages negative to the threshold for spontaneous spiking. In the absence of HCN1, the region of low or negative slope conductance observed in the voltage clamp I-V relationship prevents Purkinje cells from maintaining their membrane potential at voltages close to, but below, the threshold for spontaneous spiking.

HCN1 Stabilizes the Integrative Properties of Purkinje Cells following Inhibition of Spontaneous Spiking

The influence of HCN1 on the response to subthreshold inputs suggests that it may influence Purkinje cell output following inhibition of spontaneous spiking. To test this possibility, we applied bidirectional current ramps to Purkinje cells and calculated the modal membrane potential and mean spike frequency for 30 consecutive segments covering the ramp waveform (see Experimental Procedures) (Figure 7). The hyperpolarizing phase of the ramp shut off spiking and was used to obtain the cell's input-output relationship starting from a spontaneously active state. The depolarizing phase of the ramp was used to determine the cell's input-output relationship over the same range of injected current, but starting with the cell in a silent rather than a spontaneously spiking state.

Knockout of HCN1 had little effect on the transition

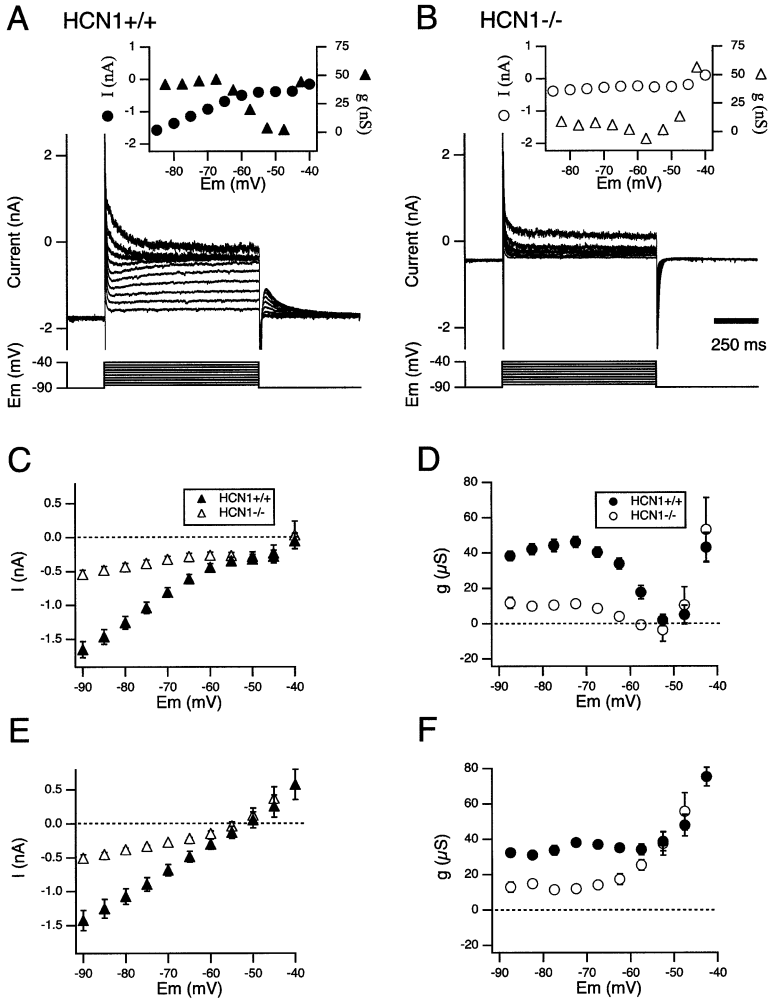

Figure 5. Purkinje Cells from HCN1$^{-/-}$ Mice Have a TTX Sensitive Region of Low or Negative Membrane Conductance at Subthreshold Potentials

(A and B) Current responses of Purkinje cells from HCN1$^{+/+}$ (A) and HCN1$^{-/-}$ (B) mice to 1 s voltage steps to potentials from −85 mV to −40 mV in 5 mV increments, from a holding potential of −90 mV. Insets plot steady-state current (circles) and slope conductance (triangles) against test potential.

(C and D) Pooled data for steady-state current-voltage (C) and conductance-voltage (D) relationships of cerebellar Purkinje cells from HCN1$^{+/+}$ (n = 9) and HCN1$^{-/-}$ (n = 5) mice. The membrane conductance of Purkinje cells from HCN1$^{-/-}$ mice at potentials between −55 mV and −70 mV is very small or negative.

(E and F) Pooled data for steady-state current-voltage (E) and slope conductance-voltage (F) relationships in the presence of TTX (1 μM) from HCN1$^{+/+}$ (n = 4) and HCN1$^{-/-}$ (n = 3) cerebellar Purkinje cells. The region of low or negative membrane conductance is abolished by TTX.

from spontaneously active to silent states, but strongly altered the membrane potential when the current was large enough to abolish spiking (Figure 7). The instantaneous frequency of the final spike on the hyperpolarizing ramp was increased slightly in the absence of HCN1 (HCN1$^{+/+}$ 10.2 ± 0.7 Hz, HCN1$^{-/-}$ 13.6 ± 1.2 Hz, p = 0.039), although the current threshold corresponding to the final spike was similar in both groups of mice (HCN1$^{+/+}$ −212 ± 22 pA, HCN1$^{-/-}$ −188 ± 21 pA, p = 0.44). Following the last spike on the hyperpolarizing ramp, Purkinje cells lacking HCN1 rapidly hyperpolarized to −92 ± 5.3 mV, whereas the peak hyperpolarization in HCN1$^{+/+}$ neurons was −66.1 ± 1 mV (p = 0.003), demonstrating the strong opposition to membrane hyperpolarization mediated by HCN1.

We observed a striking difference between Purkinje cells from HCN1$^{+/+}$ and HCN1$^{-/-}$ mice during the depolarizing phase of the ramp. The interval between the final spike during the hyperpolarizing ramp and the first spike during the depolarizing ramp was greater in HCN1$^{-/-}$ (2.04 ± 0.15 s) compared to HCN1$^{+/+}$ mice (1.29 ± 0.22 s, p < 0.05). Spiking also resumed at a >3 fold higher frequency in HCN1$^{-/-}$ (HCN1$^{-/-}$ 42 ± 5 Hz) compared to HCN1$^{+/+}$ mice (13.4 ± 1.1 Hz, p = 0.001) and with a significantly more positive current threshold for the first spike (HCN1$^{+/+}$, −203 ± 25 pA; HCN1$^{-/-}$, −68 ± 21 pA, p = 0.001). Thus, the transition of Purkinje cells from a silent to a spontaneously spiking state is characterized in the absence of HCN1 by an increased current threshold, longer delay, and higher instantaneous frequency when spiking does resume.

The transition between active and silent states of Purkinje cells from HCN1$^{+/+}$ mice was relatively independent of the prior state of activity (spiking or silent). Thus, the current threshold for the transition from spontaneously spiking to silent states was similar to the current threshold for the transition from silent to spontaneously spiking states (p = 0.79). Moreover, the instantaneous firing frequency just before firing ceased was similar to that just after firing resumed (p = 0.036). In contrast, in HCN1$^{-/-}$ mice, the transition between active and silent states was strongly influenced by the prior state of activity. The threshold current at which Purkinje cell firing ceased was significantly more negative than the threshold current at which firing resumed (p = 0.002). In addition, the instantaneous firing rate immediately before the spiking to silent transition is much lower than immediately after the silent to spiking transition (p = 0.001).

The state-dependent contribution of HCN1 to Purkinje cell excitability is clearly demonstrated by comparison

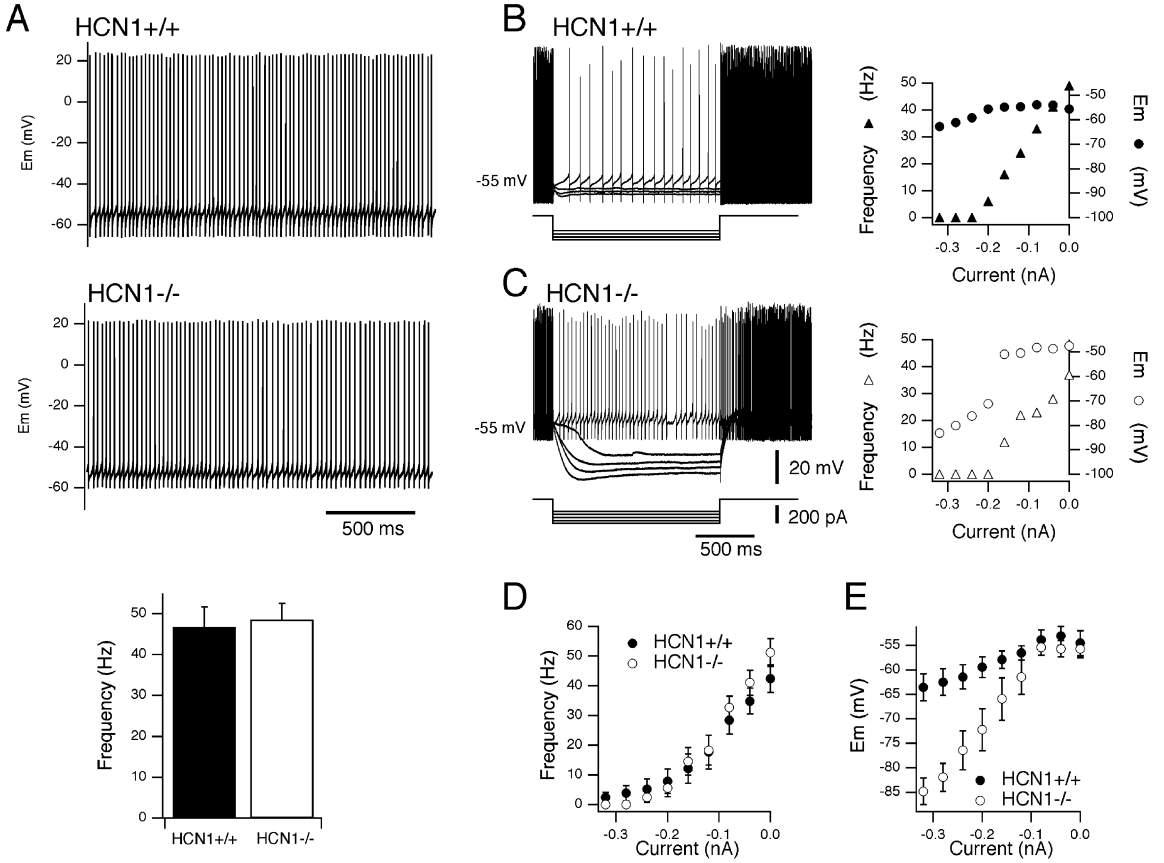

Figure 6. HCN1 Is Not Required for Spontaneous Activity in Cerebellar Purkinje Cells, but Influences Subthreshold Integrative Properties

(A) Spontaneous action potentials recorded from Purkinje cells from HCN1$^{+/+}$ (top) and HCN1$^{-/-}$ (middle) mice. The mean frequency of spontaneous action potentials is similar for Purkinje cells from HCN1$^{+/+}$ (n = 7) and HCN1$^{-/-}$ (n = 6) mice (bottom). There was also no difference between Purkinje cells from HCN1$^{+/+}$ and HCN1$^{-/-}$ mice in the threshold (-47.6 ± 0.9 mV, n = 4 versus -46.7 ± 2.2 mV, n = 3; wt versus HCN1$^{-/-}$), 10%–90% rise-time (124 ± 8 μs versus 139 ± 12 μs), peak depolarization (15.3 ± 6.4 mV versus 18.8 ± 4.2 mV), or half-width (171 ± 11 μs versus 225 ± 61 μs) of the action potential or the peak after hyperpolarization (-65.1 ± 1.1 mV versus -64.8 ± 1.9 mV).

(B) Membrane potential recordings (top) from a wild-type Purkinje cell in response to negative current steps (bottom) of amplitude up to −320 pA in 40 pA increments (left). Dependence of steady-state spike frequency and modal membrane potential (Em) on injected current (right).

(C) Recordings of membrane potential responses of a HCN1$^{-/-}$ Purkinje cell to current steps as in (B).

(D) Dependence of spike frequency on injected current is similar for Purkinje cells from HCN1$^{+/+}$ and HCN1$^{-/-}$ mice.

(E) Mean steady-state hyperpolarization in response to negative current steps is increased in Purkinje cells from HCN1$^{-/-}$ (n = 11) compared to HCN1$^{+/+}$ (n = 8) mice.

of the mean firing responses from each group of mice as a function of ramp current. The relationship between spike frequency and ramp current was independent of the direction of current change in cells from HCN1$^{+/+}$ mice, indicating that the integrative properties do not depend on whether the cell has been previously active or silent (Figures 7C and 7E). This contrasts with Purkinje cells from HCN1$^{-/-}$ mice, in which the relationship was steeper and shifted toward more positive currents when cells were activated from a silent state compared to when cell firing was shut off from an active state (Figures 7D and 7E). Similar differences were found in the relationship between average membrane potential and injected current (Figures 7F–7H). Thus, HCN1 acts to maintain the stability of the integrative properties of Purkinje cells irrespective of their preceding state of activity.

Discussion

Using a deletion knockout to investigate the cellular and behavioral functions of HCN1, we provide evidence that this channel is important for learning driven modification of motor behaviors. We also demonstrate that HCN1 enables history-independent integration of inputs in cerebellar Purkinje neurons. Although the functional expression of HCN1 is not required for a discrete behavior such as eyelid conditioning, learning of the rotarod and visible platform water maze tasks, which require more complex and repeated coordination of motor output, was profoundly impaired in HCN1$^{-/-}$ mice. Restriction of HCN1 knockout to the forebrain, but sparing the cerebellum, prevents this impairment. HCN1 was not required for spontaneous firing of Purkinje cells, which is

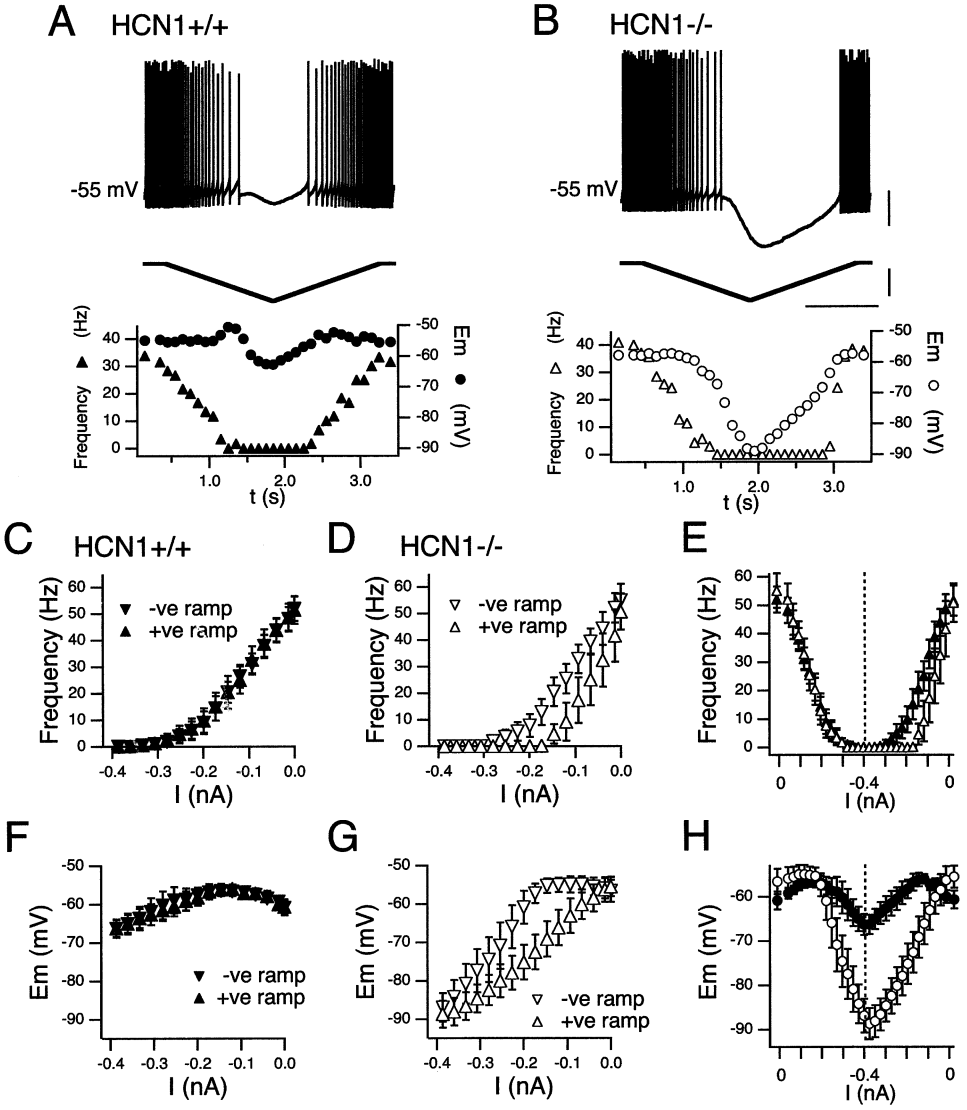

Figure 7. HCN1 Enables History-Independent Integration of Input to Purkinje Cells

(A and B) Membrane potential (top), injected current (middle), and plot of binned spike frequency (triangles) and modal Em (circles) (bottom) during current ramps, for Purkinje cells from HCN1$^{+/+}$ (A) and HCN1$^{-/-}$ mice (B). Scale bars 20 mV, 275 pA, 1 s.

(C and D) Dependence of mean spike frequency on current during the hyperpolarizing (−ve ramp) and depolarizing (+ve ramp) phase of the ramp for Purkinje cells from HCN1$^{+/+}$ (n = 7) (C) and HCN1 knockout (n = 7) (D) mice. In the absence of HCN1, the current-frequency relationship depends on the previous activity state.

(E) Comparison of mean spike frequency in Purkinje cells from HCN1$^{+/+}$ and HCN1$^{-/-}$ mice during the ramp currents. HCN1$^{+/+}$ data are indicated by closed symbols, HCN1$^{-/-}$ data are indicated by open symbols. The vertical line indicates the transition from the −ve ramp to the +ve ramp.

(F and G) Dependence of modal membrane potential on current during the hyperpolarizing and depolarizing phase of the ramp for Purkinje cells from HCN1$^{+/+}$ mice (F) and HCN1$^{-/-}$ mice (G). In the absence of HCN1, the current-voltage relationship is dependent on previous activity.

(H) Comparison of membrane potential in the two groups of mice during the ramp currents.

likely to be dependent on a resurgent sodium current (Raman and Bean, 1997, 1999). Rather, HCN1 activates upon hyperpolarization to subthreshold potentials and compensates for the decrease in membrane conductance caused by deactivation of the sodium channels. This enables the neuron to maintain an input-output relationship that is independent of previous activity.

Requirement of HCN1 for Learning and Not Execution of Complex and Rapid Movements

Cerebellar-dependent learning involves association of mossy fiber and climbing fiber inputs to the cerebellum (Albus, 1970; Kim and Thompson, 1997; Marr, 1969; Medina et al., 2002). The absence of a deficit in learning or extinction of the conditioned eye blink response indi-

cates that the synaptic pathways and plasticity mechanisms involved in this form of motor learning do not require the HCN1 channel. This is in contrast to surgical lesions to specific regions of the cerebellum or pharmacological and genetic manipulations that target synaptic transmission and synaptic plasticity, in which learning and extinction of the conditioned response are compromised or abolished (McCormick and Thompson, 1984; Medina et al., 2002; Yeo et al., 1985).

The impact of HCN1 knockout on motor learning appears to be related to the speed and repetition of movement. Thus, in the absence of HCN1, learning of a discrete motor response, such as the conditioned eyelid response, is maintained, although with modified timing. By contrast, learning of motor behaviors involving repeated movements, such as during the rotarod task, is severely impaired. Importantly, the extent of the impairment was related to the frequency of the movements. Thus, HCN1$^{-/-}$ mice fail to learn the rotarod task at a constant speed of 14 rpm, but do acquire the task at 12 rpm. Moreover, after learning the task at 12 rpm, they can maintain their balance on the rotarod at 14 rpm, strongly suggesting that the initial impairment at 14 rpm was not due to an inherent ceiling in motor performance. Rather, the HCN1$^{-/-}$ mice appear to be impaired in acquisition of the motor skills required for the higher frequency task. This also reinforces the conclusion that HCN1 is not required for execution of movements, but for learned modification of motor behaviors.

Contribution of HCN1 to Information Processing by Purkinje Cells

The spike output from a Purkinje cell is a function of its excitatory inputs from climbing fibers and parallel fibers and inhibitory inputs from basket and stellate cells. Ongoing synaptic activity modulates the frequency and pattern of spontaneous spiking and thus the output of Purkinje cells (Häusser and Clark, 1997). Integration of information coded by the activity of climbing fibers and parallel fibers occurs upon this background of tonic activity. Importantly, the intrinsic spontaneous activity of a Purkinje cell allows it to inform downstream cells of any changes in its inhibitory input relative to its excitatory input by either increasing or decreasing its spontaneous firing rate.

We demonstrate that HCN1 is required for the history-independent integration of inputs to cerebellar Purkinje cells. Thus, HCN1 activates when Purkinje cells hyperpolarize to potentials at which the resurgent sodium current deactivates. Activation of HCN1 maintains a net inward current and positive membrane conductance throughout the range of subthreshold potentials experienced by the neuron (see Figure 5). This current opposes hyperpolarization of Purkinje cells, ensuring that their membrane potential remains close to spike threshold so that small changes in membrane current do not shift the input-output relationship of the neuron (see Figures 6 and 7). As a result, in the presence of HCN1, the net effect of excitatory and inhibitory inputs at any moment is independent of the previous history of spiking activity in the cell. The Purkinje cell is therefore able to communicate information about its input reliably to downstream neurons in the deep cerebellar nuclei. This property of HCN1 also increases the effective dynamic range of Purkinje cells and reduces the time window for accurate integration of synaptic inputs.

The function we describe for HCN1 will only be important in vivo if hyperpolarizing inputs of sufficient magnitude to abolish spontaneous spiking and engage HCN1 actually occur. Several observations indicate that this is likely to be the case. Spontaneous Purkinje cell firing in vivo is abolished by inhibitory pathways activated by parallel fiber stimulation (Andersen et al., 1964). Inhibition of Purkinje cell firing also occurs during specific motor behaviors. During conditioned eye blink responses, Purkinje cell activity consists of an initial increase followed by a reduction in spike frequency (Hesslow and Ivarsson, 1994). During alternating limb movements, inhibition of Purkinje cell spiking is correlated with flexed or extended limb positions (Thach, 1968). Repetitive alternating movements involved in running on the rotating wheel or swimming in the watermaze are likely to involve similar phasic changes in Purkinje cell activity. Thus, the integrative properties of Purkinje cells may become compromised during pauses in spiking associated with specific phases of these behaviors.

Might other classes of neurons contribute to the motor learning deficit? Although our data suggest that HCN1 in Purkinje cells may be important for motor learning, as HCN1 is expressed in other populations of neurons, it is not possible to definitely conclude that the absence of HCN1 from Purkinje cells causes the motor learning deficit. Since restriction of HCN1 knockout to the forebrain prevented the rotarod impairment, the deficit is unlikely to involve neurons in the cerebral cortex or striatum. Motor learning pathways in the basal ganglia are also unlikely candidates, as they are utilized for reward-based learning rather than optimization of the timing of movements such as those required for the rotarod task (Doya, 2000). However, deficits in other parts of the olivo-cerebellar circuit may contribute to the learning impairment. For example, HCN1 is also expressed by cerebellar basket cells and by neurons in the inferior olive. Our initial investigations indicate that knockout of HCN1 does alter the spontaneous firing properties of a subpopulation of molecular layer interneurons, but this results in only small changes in the spike firing pattern of a minority of Purkinje cells, and no change in overall spike frequency or coefficient of variation (unpublished observations). This subtle indirect change contrasts with the profound direct effect of HCN1 knockout on all Purkinje cells described here. Although expression of HCN1 in the inferior olive is modest compared with other regions, including the Purkinje cell layer (Santoro et al., 2000), changes in the inferior olive could also alter motor learning in HCN1$^{-/-}$ mice. Further discrimination of the role of HCN1 in distinct populations of neurons will require more spatially restricted manipulation of the channel.

A Model of the Role of HCN1 in Purkinje Cells during Motor Learning

Can the electrophysiological deficit that we characterize in Purkinje cells contribute to the impaired motor learning and memory caused by knockout of HCN1? Based on the requirement for HCN1 to maintain history inde-

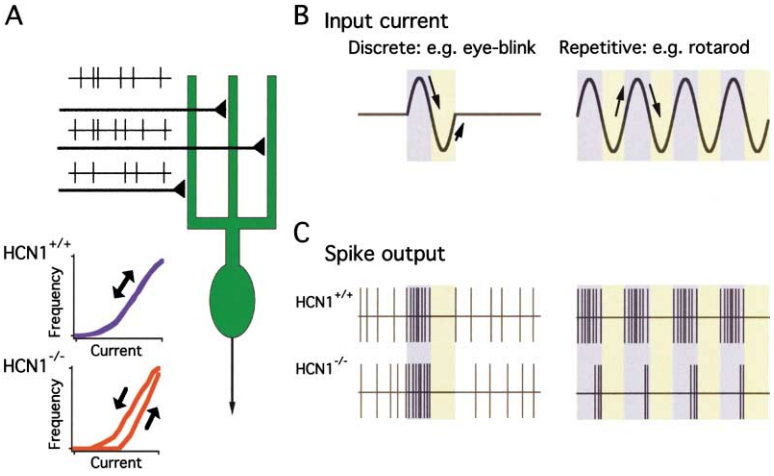

Figure 8. A Model for the Role of HCN1 Channels in Motor Learning through History-Independent Integration of Inputs by Cerebellar Purkinje Cells

(A) Presynaptic spike trains evoke synaptic currents in Purkinje cells that are integrated to produce output spikes. The input-output function of HCN1$^{+/+}$ Purkinje cells (blue, from Figure 7) is independent of the history of activity. In contrast, the input-output function of Purkinje cells from HCN1$^{-/-}$ mice (red, from Figure 7) depends on whether the neuron is initially in an active (down arrow) or silent (up arrow) state.

(B) Schematized patterns of net synaptic input to Purkinje cells during discrete motor behaviors, such as eyelid conditioning, and during repetitive motor behaviors, such as the rotarod task.

(C) Schematic of the predicted effects of HCN1 knockout on spike output of Purkinje cells to the inputs in (B). During repeated movement, we suggest that Purkinje cells without HCN1 would no longer produce output patterns appropriate to the phase of the movement. This would have two consequences. First, synaptic plasticity underlying motor learning may not be induced, for example during the prolonged recovery from inhibition (Ekerot and Kano, 1985), preventing the modification of synapses underlying motor learning. Second, the output of the cerebellar cortex in response to a given input pattern may no longer appropriately modify motor output.

pendent integration in Purkinje cells, we suggest a model in which the different effects of HCN1 knockout on motor learning are explained by the different temporal properties of the inputs to Purkinje cells during each behavior (Figure 8). Thus, during eye blink conditioning, pauses in Purkinje cell output (Hesslow and Ivarsson, 1994) are thought to lead to disinhibition of deep cerebellar neurons and initiation of the conditioned eye blink response (Medina et al., 2002). We suggest that the absence of HCN1 will not significantly modify the onset of the pause in spiking, and therefore conditioning can occur (Figures 8B and 8C). However, the silent period may be extended, thereby prolonging the disinihibiton of deep cerebellar neurons, a change that could lead to the increased expression of the conditioned response at short latencies by modifying the induction of synaptic plasticity in these neurons (Aizenman and Linden, 2000; Aizenman et al., 1998).

The rotarod task, unlike eye blink conditioning, requires accurate repetition of a series of similar movements. Silent periods occur in the firing of Purkinje cells during specific phases of behaviors that involve repeated contraction and relaxation of specific muscle groups (e.g., Thach, 1968), indicating that inhibition dominates the input to Purkinje cells during specific phases of movement (Figure 8B). We suggest that during the rotarod task, HCN1 functions to stabilize the input-output relationship of Purkinje cells during these silent periods (Figure 8C). We propose that in the absence of HCN1, motor learning may be impaired through either of two mechanisms. First, during motor learning, prolonged hyperpolarization may prevent induction of LTD at parallel fiber synapses (Ekerot and Kano, 1985). Second, during execution of motor behaviors, Purkinje cells lacking HCN1 may be unable to transform synaptic inputs occurring after a silent period into the appropriate spike patterns required for useful modification of motor output by the cerebellum. According to this proposed model, in the absence of HCN1, the short intervals between successive phases of high frequency movement will be insufficient for recovery of the Purkinje cell integrative properties from inhibition, resulting in impaired function. Longer intervals between successive phases of lower frequency movement may be sufficient to allow recovery of the Purkinje cell integrative properties, even in the absence of HCN1. This provides a potential explanation for the observed frequency dependence of the rotarod impairment. A critical test of this proposed model will be to record directly from Purkinje cells during motor learning tasks.

One of the main results of this study is that specific alterations in the nonsynaptic excitable properties of neurons can have a pronounced impact on memory function. In the past, a major emphasis in the study of learning has been placed on the importance of synaptic plasticity as the site of memory storage. Our study emphasizes that the active integrative properties of a neuron, as determined by the expression of specific ion channels, in this case HCN1, are critical for appropriate learning and memory of motor behaviors. Our study also suggests that regulation of ion channel activity, in this case by genetic deletion, but also potentially by neurotransmitters that modulate channel function (Li et al., 1993), is able to exert strong control over behavior. In conclusion, the correct tuning of a neuron's excitable properties may be necessary for the induction or expression of synaptic plasticity involved in complex behaviors, including the learning of motor tasks.

Experimental Procedures

Generation and Genotyping of Mice with Complete and Restricted KO of HCN1

Gene targeting in the ES stem cells was performed in two steps (Supplemental Figure S1A at http://www.cell.com/cgi/content/full/115/5/551/DC1). During the first step of homologous recombination, a LoxP site was inserted upstream of the P region and S6 encoding exon, and a cassette containing thymidine kinase and neomycin-resistant genes flanked by LoxP sites was inserted downstream of

this exon. During the second step, Cre recombinase was transiently expressed in the targeted clones producing two types of clones. The first one, lacking the selectable cassette and the exon encoding for the pore and S6 region, gave rise to a conventional knockout of HCN1. The second type of clone, lacking the selectable cassette and containing a floxed pore and S6 encoding exon (HCN1f allele), was used for obtaining a restricted knockout of HCN1.

Genetic deletion of the pore and S6 region was confirmed by Southern blot analysis (Supplemental Figure S1B–S1E). In situ hybridization demonstrated expression patterns for HCN1 in the brains of wild-type and $HCN^{f/f}$ mice similar to those previously described (Santoro et al., 2000). The mRNA encoding the pore and S6 transmembrane domain of HCN1 was completely absent from brains of $HCN1^{-/-}$ mice, whereas this deletion was restricted to neurons in the forebrain of $HCN1^{f/f,cre}$ mice (Supplemental Figure 2A).

Both $HCN1^{-/+}$ and $HCN1^{f/+}$ mice were maintained on a 129SVEV background. For experiments with the complete knockout, 129SVEV $HCN1^{-/+}$ animals were crossed with C57 wild-type mice and their hybrid $HCN1^{-/+}$ progeny were intercrossed to produce $HCN1^{-/-}$ and $HCN1^{+/+}$ littermates. For the restricted knockout experiments, the $HCN1^{f/f}$ 129SVEV/C57 hybrid males were crossed to $HCN1^{f/f,cre}$ females obtained by crossing $HCN1^{f/+,cre}$ mice on a C57/129SVEV hybrid background with $HCN1^{f/f}$ 129SVEV/C57 hybrid males. The $HCN1^{f/f,cre}$ and $HCN1^{f/f}$ littermates with mixed average 50%/50% 129SVEV/C57 background were used in experiments. The presence of the cre allele was determined by Southern hybridization using a cre-specific probe.

For in situ hybridization, mouse brains were dissected and frozen in OCT embedding medium. 20 μm sections were prepared and hybridized as described (Huang et al., 1999) to an [a-35S] dATP-labeled HCN1 anti-sense oligonucleotide: GCCCACAATCATGCT CAGCATGGTAATCCAGAGGTCAGACATGCTGAC.

Western blotting was performed following dissection of total brain or isolated hippocampus, cortex, and cerebellum membranes from adult mice (Supplemental Figure 2). Antibodies against the HCN1 C-terminus and HCN2–4 were generously provided by Dr. B. Kaupp and Dr. F. Mueller (Stevens et al., 2001).

Behavior

Eye blink, ear twitch, rolling, and righting reflexes were tested using standard procedures (Paylor et al., 1999). The hot water tail flick test measures the latency for a mouse to withdraw its tail when immersed in a beaker of water maintained at 55°C (Fairbanks and Wilcox, 1997). The latency to the first rapid tail flick was measured.

The watermaze task was performed as described previously (Malleret et al., 1999) with two training phases: 2 training days with a visible platform, followed by four training days with a hidden platform. For each training phase, 4 trials, 120 s maximum duration and 15 min inter-trial interval, were given daily. The probe trial was 60 s in duration. The trajectory of each animal was recorded with a video tracking system (HVS Image Analyzing VP-118).

The accelerating rotarod apparatus (Ugo Basile) was used to measure motor coordination. During the training period, mice were placed on the rotarod starting at 5 rpm and slowly accelerating to 44 rpm. The maximum observation time was 5 min. Animals were trained for 3 consecutive days, receiving 4 trials per day with a 1 hr intertrial interval. During testing on the fourth day animals received seven consecutive 2 min trials at constant speeds of 44, 33.7, 31, 24.5, 18, 11.5, and 5 rpm. During the constant speed rotarod experiment, animals were also given four trials per day. The latency to fall during the observation period was recorded. Rotarod data was analyzed with ANOVA statistical tests. Post hoc comparisons used the planned comparison test.

Eyelid conditioning was performed on male $HCN1^{+/+}$ ($n = 12$) and $HCN1^{-/-}$ ($n = 12$) mice, 2–3 months of age, housed individually with 12 hr light/dark cycle and ad libitum access to food and water. Under ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) anesthesia, 4 wires (2 for recording EMG and 2 for delivering shock) were implanted subcutaneously at the left upper eyelid. Mice were trained with the experimenter blind to their genotype. After 1 day of habituation, the mice underwent 7 days of delayed eyelid conditioning followed by 5 days of tone-alone extinction. The conditioned stimulus (CS) was a 352 ms tone (1 kHz, 85 dB, 5 ms rise/fall time) and was adjusted daily to maintain the same decibel level. The unconditioned stimulus (US) was a coterminating 100 ms shock (100 Hz biphasic square pulse) and was adjusted daily for each mouse to elicit a small head turn response with a minimal voltage. There was a 252 ms interstimulus interval and a randomized intertrial interval between 20–40 s (average 30 s). The daily paired training consisted of 100 trials grouped in 10 blocks. Each block consisted of a tone-alone (1^{st}) trial, a shock-alone (6^{th}) trial, and 8 paired (2^{nd}–5^{th}, 7^{th}–10^{th}) trials. During extinction all trials were shock-alone trials.

The criteria for determining valid CR trials were the same as in previously studies (Chen et al., 1995; Shibuki et al., 1996). Three types of trials were excluded from daily % CR performance: (1) high EMG activity before the CS onset: when the average unit count per bin was higher than 2 during the 252 ms pre-CS period; (2) unstable EMG activity before the CS onset: when the SD of unit counts per bin was larger than the average unit count per bin; and (3) short-latency startle response to the CS: when the average unit count within 28 ms after the CS onset was bigger than the average plus the SD of the pre-CS period. For paired trials, the CR period (168 ms) was set from 84 ms after the tone onset until 252 ms (just before the shock onset). For tone-alone trials, the CR period was extended to the termination of the tone (168 ms + 100 ms = 268 ms). In both paired and tone-alone trials, the CR was defined as the average unit count of any consecutive 28 ms during the CR period that was higher than average plus SD plus 1 unit per bin of pre-CS period. The average, standard deviation, and standard error of the mean of daily % CR were calculated with Excel (Microsoft). CR peak latency was analyzed with Student's t test and the Kolmogorov-Smimov test of SPSS. CR performance of acquisition and extinction periods was analyzed with ANOVA using Statistica (Statsoft).

Electrophysiology

Electrophysiological recordings were made from sagital cerebellar slices prepared from 3- to 8-week-old mice. Mice were decapitated and their brains rapidly removed and placed in cold (2°C) modified ACSF of composition (mM), NaCl (86), NaH_2PO_4 (1.2), KCl (2.5), $NaHCO_3$ (25), glucose (25), $CaCl_2$ (0.5), $MgCl_2$ (7), and sucrose (75). The middle cerebellar vermis was dissected out, glued to an agar block, and cut submerged under cold modified ACSF into 200 μm sections with a Vibratome 3000 system. Slices were transferred to a storage container filled with standard ACSF at 33–35°C for 30–40 min and then allowed to cool to room temperature (20–22°C). The standard ACSF had the following composition (mM), NaCl (124), NaH_2PO_4 (1.2), KCl (2.5), $NaHCO_3$ (25), glucose (20), $CaCl_2$ (2), $MgCl_2$ (1). For recording, slices were transferred to a submerged chamber and Purkinje cells were visually identified with DIC optics under infrared illumination. Slices could be maintained in a condition suitable for satisfactory quality recordings for a maximum of six hours after the dissection. I_h was recorded from Purkinje cells at room temperature under conditions designed to minimize the contribution of other voltage-gated currents. The ACSF for recording I_h had the composition (mM): (NaCl (115), NaH_2PO_4 (1.2), KCl (5), $NaHCO_3$ (25), glucose (20), $CaCl_2$ (2), $MgCl_2$ (1), $BaCl_2$ (1), $CdCl_2$ (0.1), 4-AP (1), TEA (5), NBQX (0.005), bicuculline (0.02), and TTX (0.0005). All other electrophysiological experiments were performed at 33–35° in the absence of pharmacological agents, unless stated otherwise.

Whole-cell recordings were obtained from Purkinje cell somata with 2–5 MΩ resistance electrodes filled with intracellular solution of composition (mM), KMethylsulfate (120), KCl (20), HEPES (10), $MgCl_2$ (2), EGTA (0.1), Na_2ATP (4), Na_2GTP (0.3), and phosphocreatine (10). Series resistances were ≤15 MΩ for voltage clamp experiments and ≤25 MΩ for current clamp experiments. There was no significant difference between the series resistance of recordings between $HCN1^{+/+}$ and $HCN1^{-/-}$ mice in either configuration. Series resistance in voltage clamp recordings was compensated by 50%–80%. For current clamp recordings, appropriate bridge and electrode capacitance compensation were applied. Membrane current and voltage were filtered at 1–2 KHz and 4–20 KHz and sampled at 5–10 KHz and 10–50 KHz for voltage and current clamp experiments, respectively. In some figures, action potentials are truncated due to undersampling of the rapid membrane potential change during the action potential. For analysis of action potential waveforms, a 20 KHz filter and sample rate of 50 Khz were used to allow adequate sampling of the membrane potential.

Data were analyzed using custom-written routines in IGOR pro (Wavemetrics). Modal membrane potential was calculated after sorting the membrane potential at each time point sampled into 0.2 mV bins. For construction of steady-state current voltage relationships, the modal membrane potential and mean spike frequency were calculated from the final 1 s of each step. For analysis of ramp currents, 5–10 consecutive responses were analyzed. Data during each ramp was divided into 30 segments for which the mean across all responses of the modal membrane potential and spike frequency were calculated. Analysis of ramp waveforms with lengths of 3 s or 6 s gave similar results, and data were therefore pooled when appropriate. Statistical analysis was performed using the appropriate Student's t test.

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